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Down-regulation of Lysyl Oxidase-induced Tumorigenic Transformation in NRK-49F Cells Characterized by Constitutive Activation of Ras Proto-oncogene*

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Several investigations have suggested a putative tumor suppressor role for lysyl oxidase because it is down-regulated in many human and oncogene-induced tumors. To address this issue we down-regulated the enzyme in normal rat kidney fibroblasts by stable transfection of its cDNA in an antisense orientation. The selected clones revealed an absence of lysyl oxidase and dramatic phenotypic changes, interpretable as signs of transformation. The antisense lysyl oxidase clones showed, indeed, loose attachment to the plate and anchorage-independent growth and were highly tumorigenic in nude mice. Moreover, we found an impaired response of the PDGF and IGF-1 receptors to their ligands. In particular, the transformed cells showed a down-regulation of both PDGF receptors and expressed the 105-kDa isoform of the IGF-1β receptor, which was not present in the normal control cells. The lack of response to PDGF-BB has been described as a feature of many *ras*-transformed phenotypes. Therefore, we looked at the status of the $p21^{ras}$. Indeed, we found a significantly higher level of active $p21^{ras}$ both during steady-state growth and prolonged starvation. Our data reveal new evidence for a tumor suppressor activity of lysyl oxidase, highlighting its particular role in controlling Ras activation and growth factor dependence.

Lysyl oxidase (LOX)[1] (protein-6-oxidase; EC 1.4.3.13) is the key enzyme that controls collagen and elastin maturation (1, 2). Indeed, it catalyzes the oxidative deamination of peptidyl lysine and hydroxylysine to peptidyl-α-aminoadipic-δ-semialdehyde into elastin and collagen chains. The consequent aldehydes lead to a spontaneous condensation forming inter- and intrachain cross-links. This post-translational modification of extracellular matrix molecules seems to have a very important role both for collagen and elastin structural aspects and for triggering still unknown signal transduction pathways. Several reports have suggested a clear association between organ fibrosis and increased LOX activity (3–9).

The most intriguing aspect regarding LOX activity refers to its putative cell phenotype control and/or tumor suppressor activity. In many naturally occurring and oncogene-induced tumors, LOX is down-regulated, while, in contrast, LOX is one of the main genes induced in concomitance with the reversion process (10–14). In particular it seems that LOX was down-regulated in cells transformed by *ras* or *ras*-dependent oncogenes, so that it was first identified as a "*ras* recision gene" (*rrg*) (10, 11, 13). In particular, Friedman and co-workers (10, 11) showed that H-*ras*-transfected NIH-3T3, induced to revert by interferon β/γ, would return to their transformed phenotype upon transfection with an antisense LOX vector. The reversion or the re-transformation did not affect the level of $p21^{ras}$ although other possible mechanisms or parameters were not studied (10).

The localization of the enzyme is mainly extracellular, although recently it has been confirmed that processed LOX is localized intracellularly and inside the nucleus (16–18). Our recent finding that LOX can enhance the transcriptional activity of the *COL3A1* promoter (15) seems to suggest a direct function for LOX in the nucleus. Therefore, LOX may have an intracellular substrate(s) that mediates its ability to control the cell phenotype. Despite these intriguing findings, there are no hypotheses to date about the mechanism through which LOX might actually work as a tumor suppressor. In the present study we have addressed this issue by studying the effects of the down-regulation of LOX in normal rat kidney fibroblast cells (NRK-49F).

MATERIALS AND METHODS

Antisense Vector and Transfection—NRK-49F cells were stably transfected with pCLO8 vector, a pCDNA3 plasmid carrying the fragment from −33 to +995 of the mouse LOX coding sequence (19) in antisense orientation subcloned in *Kpn*I and *Xba*I restriction sites. A control transfection on the same cells was performed with the pCDNA3 vector alone. Both transfections were performed in quadruplicates. Several pCLO8 clones (as-LOX) were isolated after extensive selection with G418 and designated as DDM-AL-A/E/C/D for those derived from the antisense LOX-transfected plates. The controls were named DDM-A/B/C/D. From the clones A/B/C/D of both transfections further clones were selected and designated by a number following the letter of the original clone (e.g. A1, A2,...C4).

Cell Culture—NRK-49F were grown in Dulbecco's modified Eagle's medium, 10% fetal calf serum (FCS), 1% glutamine, 1% nonessential amino acids, and antibiotics at 37 °C, 5% CO_2 in a humidified incubator. The clones derived from the transfection with pcDNA3 and pCLO3 plasmids were selected by adding 400 μg/ml G418 to the above medium for at least a month. K-NRK, normal rat kidney fibroblasts transformed by K-*ras* (American Tissue and Cell Culture (ATCC), Manassas, VA), were grown in the same medium as above. LPS8–3 cells, NIH-3T3 fibroblasts transformed by Ha-Ras^{Val-12}, were grown in the same medium as above, but with 1% pyruvate and without nonessential amino

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[1] The abbreviations used are: LOX, lysyl oxidase; LOL, LOX-like; NRK, normal rat kidney; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; FCS, fetal calf serum; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor.

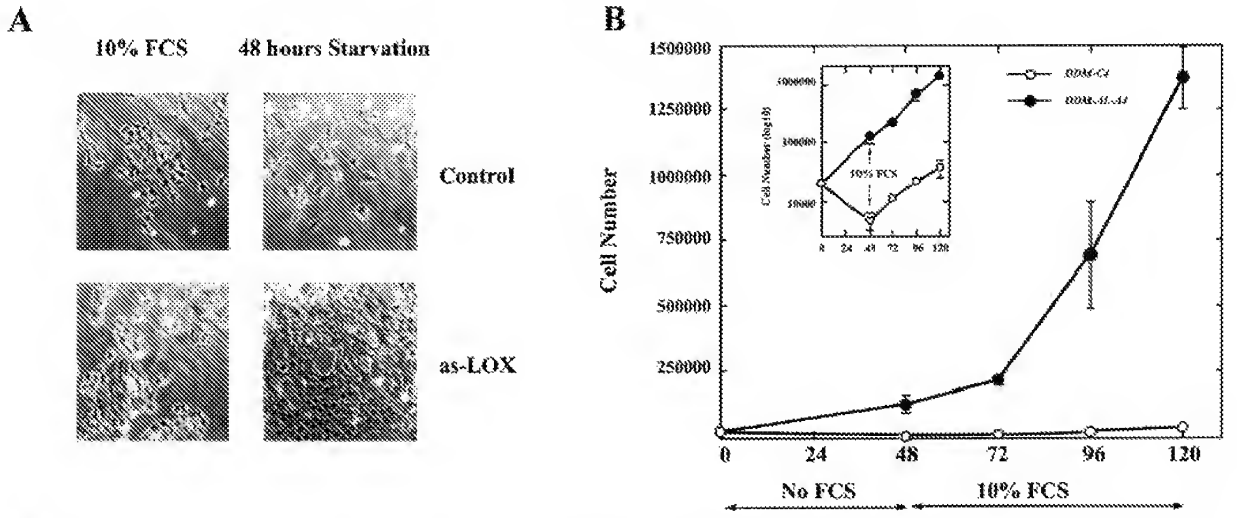

FIG. 1. *A*, phenotype comparison between the control cells, NRK-49F transfected with vector alone (*upper panels*) and as-LOX cells (*lower panels*). In the *left panels* control-transfected NRK-49F cells (DDM-C4) and pCLO3/as-LOX clone DDM-AL-A4 were grown in their normal medium containing 10% FCS. In the *right panels* the same cells were grown for 48 h without 10% FCS (*starvation*). Notice the increase in the cell density for as-LOX even after starvation (compare *lower panels, left* and *right*). *B*, graph showing the growth in control cells (*DDM-C4*) and as-LOX cells (*DDM-AL-A4*). About 20,000 of the indicated cells were seeded in 35-mm plates in their normal culture conditions. After adhesion to the plate (24 h), the cells were deprived of FCS for 48 h. Subsequently the growth medium was re-integrated with 10% FCS, and the cells were grown for 3 more days. The same results are shown in the *inset*, but using a logarithmic scale to express the number of cells. This allows the detection of the dramatic cell growth arrest and death in the control cell line during the 48 h of starvation. The results expressed are the average ± S.E. of a typical experiment performed in triplicate.

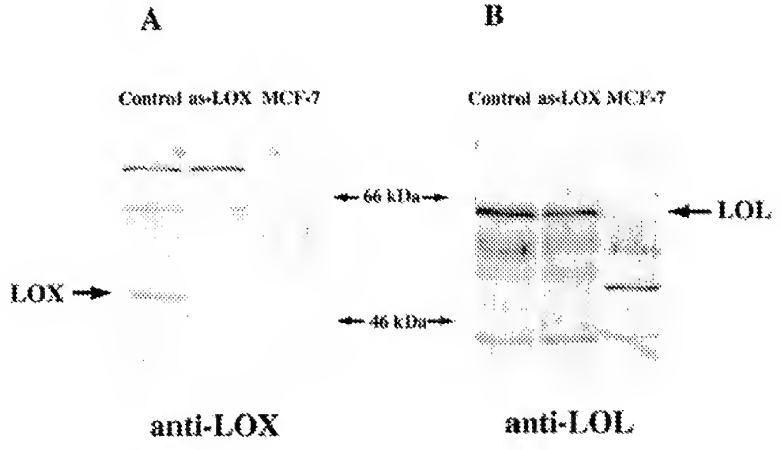

FIG. 2. **LOX and LOL protein expression in control and as-LOX cells.** 30 μg of total cell lysates from the indicated cell lines were analyzed by Western blot with anti-LOX (*A*, Ref. 7) and anti-LOL (*B*, Ref. 22) rabbit polyclonal. In the figure, the *bands* corresponding to LOX and LOL precursors are indicated. The as-LOX clone used in this experiment was DDM-AL-A4, but similar results were obtained with almost all the tested as-LOX clones.

acids (kindly provided by Dr. Juan Carlos Lacal, Instituto de Investigaciones Biomédicas, CSIC, Madrid, Spain). The MCF-7 cells are derived from a mammary carcinoma tumor (ATCC) and were grown in the same medium as for NRK-49F, but without nonessential amino acids.

Anchorage-independent Colony-forming Assay—About 5×10^3 cells/35-mm plate were seeded in 0.35% top agarose and poured over a layer of 0.5% agarose. Both agarose layers were prepared to contain 1× of the medium required for the indicated cells. The plates were incubated at 37 °C, 5% CO_2 under humidified conditions.

Tumorigenicity of as-LOX Cells in Nude Mice—5-week-old nude mice clone crl:cd1-nuBR (Charles River, Lecco, Italy) were injected subcutaneously with 10^6 cells from the following cell lines: controls, NRK-49F, and DDM-C4 (NRK-49F transfected with pCDNA3.1 alone); as-LOX, DDM-AL-A4 and -C6; positive control, LPS-3. The mice were kept under standard sterile conditions and followed for the indicated times. The three dimensions, height (h), length (l), and width (w), of each tumor were measured at the indicated times, and the volumes were calculated according to the following formula: volume = $\{\pi h(h^2 + 3a^2)\}/6$, where a = (w + l)/4 (20). To test the recidivism of the primary neoplasias, tumors from one control individual and two from the as-LOX groups were excised. Then the animals were followed up for 3 weeks.

Protein Analysis—Total cell lysates were prepared in radioimmune

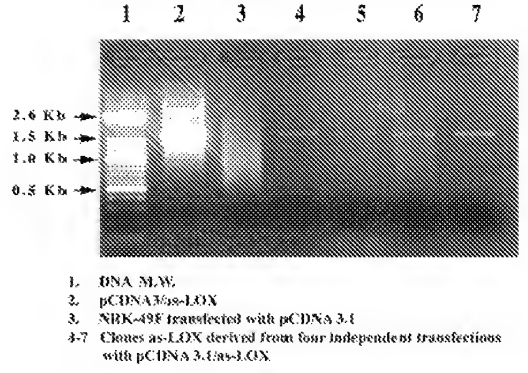

FIG. 3. **PCR detects the insertion of pCLO3 in as-LOX clones.** The figure shows that the expected 1.6-kilobase band is amplified only from the genomic DNA of the as-LOX cells, whereas the control cell DNA produced only a smear. *Lane 2* shows the expected amplification product using 10 ng of pCLO3 as template.

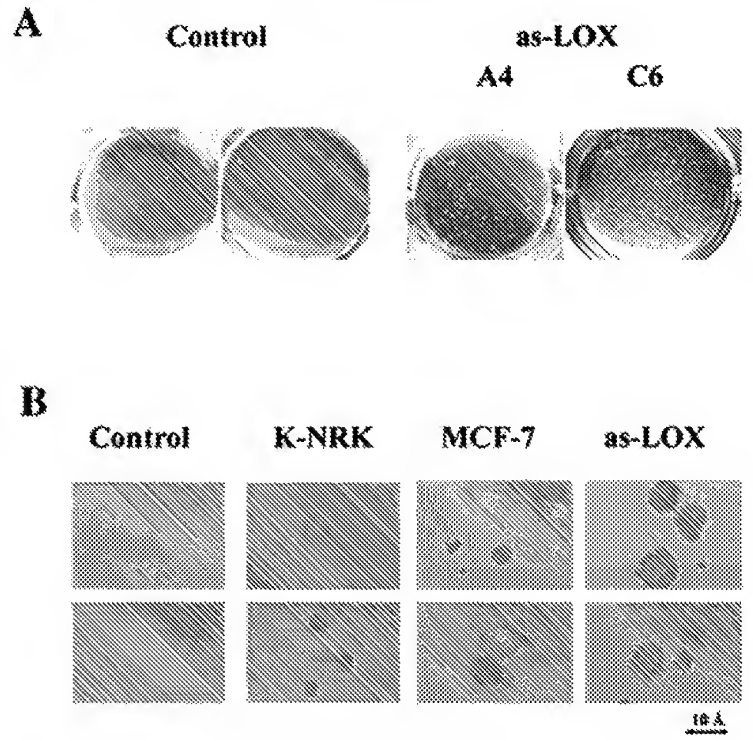

FIG. 4. **Anchorage-independent colony-forming assay.** *A*, macroscopic view of the colonies formed by the indicated cells. *B*, microscopic view of the colonies formed by the indicated cells. Controls cells are NRK-49F transformed with pcDNA3 vector alone. The as-LOX clones shown in the picture were DDM-AL-A4 and -C6. In as-LOX and MCF-7 plates, the colonies appeared after 8–10 days. The micrographs were taken at × 40 magnification.

precipitation buffer, containing a protease mixture and the phosphatase inhibitor I and II mixtures (Sigma). The lysates were cleared by 30-min centrifugation at 30,000 × *g*. Typically, 30 μg of the total cell lysate were separated on SDS-PAGE (21). Then the gels were analyzed by Western blot with the commercial antibodies indicated in the respective figures. Anti-LOX and anti-LOL rabbit polyclonal have been previously described (7, 22). The recognized proteins were detected by using a secondary anti-rabbit antibody coupled to alkaline phosphatase and developing the blot with NBT/BCIP reagents (Roche Molecular Biochemicals, Mannheim, Germany). The immunoprecipitations were performed starting from 600 μg of total cell lysates, which were diluted to 600 μl with StaphA buffer, containing 8.6 mM Na_2HPO_4, 1.6 mM NaH_2PO_4, 0.1 M NaCl, 1% Triton X-100, 0.1% SDS, 16 mM NaN_3. The samples were first precleared with protein G-agarose resin (Roche Molecular Biochemicals) and then incubated with the indicated antibodies for 2 h in an ice-cold bath. Finally, they were incubated overnight with 50 μl of protein G-agarose at 4 °C under vigorous shaking. The next day the resin was washed at least four times with StaphA plus 1% bovine serum albumin (Sigma). The washed resin was finally resuspended in 20 μl of Laemmli buffer and loaded onto SDS-PAGE for further Western blot analysis.

Polymerase Chain Reaction (PCR)—To verify that the as-LOX cells carried the expression vector, we performed PCR to detect the plasmid, using as template the genomic DNA extracted from four independent as-LOX clones. The primers were designed to amplify a fragment overlapping the plasmid and the antisense LOX sequence. The forward primer, specific for pCDNA3.1 vector, was in position 707: 5′-GCAAATGGGCGGTAGGCGTGTAC-3′; the reverse primer was specific for the antisense LOX sequence and designed in position 2304: 5′-GTCACGCTGCGCGTAACCACCACA-3′. The expected product was about 1.6 kilobases. The samples were amplified for 32 cycles with an annealing at 58 °C for 30 s, and the extension step was at 72 °C for 1 min. In the incubation buffer supplied by the manufacturer (Roche Molecular Biochemicals) 5% dimethylsulfoxide was added.

RESULTS AND DISCUSSION

The lysyl oxidase antisense clones (as-LOX) clones showed striking phenotypic changes when compared with the control clones (Fig. 1*A*). The Western blot analysis (Fig. 2*A*) showed a dramatic down-regulation of LOX in the as-LOX clone (DDM-AL-A4), where the protein was practically undetectable. The same results were obtained in many other as-LOX clones, proving that LOX down-regulation was not the random product of a genomic insertion effect (data not shown). We confirmed with PCR the presence of the transfecting vector pCLO3 integrated in their genomic DNA (Fig. 3). Fig. 2*B* also showed that the antisense sequence did not block the translation of LOX-like (LOL) messenger, the most LOX-related among the other components of the LOX gene family (23–26). as-LOX cells showed a faster and more continuous growth with confluence reached at a number of cells that was higher than the controls (Fig. 1*B*) and a looser attachment to the plate. Moreover, we noticed a stronger ability to grow under starvation conditions for more than 48 h, whereas the control clones showed a typical sufferance evident from the reduction of the cytosolic compartment and elongation of the cell body (Fig. 1*A*). The same picture shows that the density of the as-LOX cells increased despite starvation, whereas there was no growth in the control cells, and cell death was evident (Fig. 1, *A* and *B*). These observations prompted us to look for the typical features of tumorigenicity. Fig. 4, *A* and *B* shows a soft-agar colony-forming assay that tests the ability of the cells to grow under anchorage-independent conditions. The experiment also included two positive controls, K-NRK cells, K-*ras* oncogene-transformed homologous to NRK-49F, and a mammary tumor cell line, MCF-7. In Fig. 4*A*, a macroscopic comparison of the colony-forming ability of two control clones *versus* two as-LOX clones selected from two independent transfections is shown. It is clear that as-LOX can grow in soft-agar forming a great number of large colonies, whereas the control cells formed fewer and smaller colonies, almost undetectable to the naked eye. As expected, K-NRK and MCF-7 cells also formed colonies, although K-NRK to a lesser extent. Fig. 4*B* shows a microscopic image of the colonies formed by the tested cell lines. It appears that as-LOX clones and MCF-7 produced comparable size colonies. As a further approach to define the transformed status of as-LOX cells, we tested their tumorigenicity when injected in nude mice. We injected subcutaneously 10^{-6} cells/mouse in a total of 14 ani-

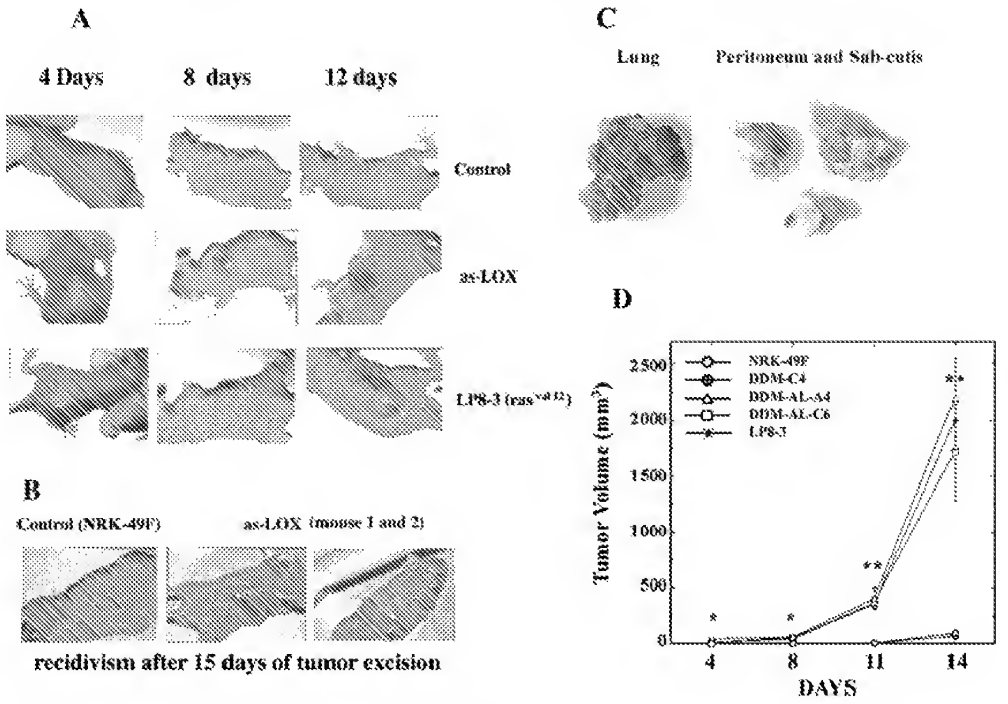

FIG. 5. *A*, tumorigenicity of as-LOX cells in nude mice. 5-week-old nude mice were injected subcutaneously with 10^6 cells of the indicated cell lines (see "Materials and Methods" for details). The picture shows a typical individual of each group. There was no difference between NRK-49F and DDM-C4 as controls and between DDM-AL-A4 and -C6 as as-LOX cells. Each group was composed of 14 animals used in three independent experiments. *B*, recidivism of the primary tumors in as-LOX-injected mice. The tumors from one individual of the control group and two of the as-LOX group were extensively excised, and the wounds were reclosed under sterile conditions. The animals were followed-up until the tumors reappeared in the as-LOX mice after 2 weeks. Nothing was detected in the control mouse during the following 1 week of follow-up. *C*, metastasized internal organs: lungs, pleura, and subcutis nodes from the recidivant mice. *D*, time-course of tumor development. The volumes measured at the indicated times were calculated as described under "Materials and Methods." The graph reports the average tumor volume from each group ± S.E. The significance of the differences between as-LOX and control cells was evaluated by Student's *t* test and is indicated in the graph.

mals for each cell line tested. We used two selected as-LOX clones, DDM-AL/A4 and C6, and a NIH-3T3 cell line transformed by Ha-*ras*$^{Val-12}$ (LP8–3) (27) as a positive control. We used NRK-49F cells transfected with the vector alone as well as the parental NRK-49F cells as negative controls. The mice injected with either as-LOX clones had a strong and extremely rapid tumorigenic response (Fig. 5*A*). Subcutaneous nodules were already detectable after four days from the injection. The negative controls did not show any signs of tumor. The mice injected with LP8–3 cells also developed small tumors during the first week, and between day 8 and 14 they developed tumors comparable in size to the ones formed in the as-LOX-injected mice. The histology showed that the tumors developed by as-LOX cells were classical fibrosarcomas (data not shown). Late in the experiment, 5 of 28 control mice developed some very small tumors. A potential tumorigenicity of NRK-49F is also mentioned in the American Tissue and Cell Culture catalog and seems to be suggested also by those little colonies developed in our soft-agar experiments (Fig. 4, *A* and *B*). Moreover, to test the metastatic potential of the tumors developed in the as-LOX-injected mice, we excised the tumors in two animals from the group and followed them up for recidivism. After 10–15 days from the excision of the primary tumor, both animals developed a very aggressive secondary tumor, witnessing a high metastatic potential (Fig. 5*B*). In fact, in the autopsy of the two tested animals, we found a massive infiltration of the peritoneum and lungs (Fig. 5*C*). We submitted one of the control animals, which had developed a small tumor, to the same procedure, but it did not show recidivism during the tested time. Fig. 5*D* shows a graphical comparison of tumor development among the tested cell lines. The tumorigenicity induced by LOX down-regulation is striking and comparable with the one induced by the NIH-3T3 cells expressing the activated *ras*-proto-oncogene (LP8–3).

In an attempt to characterize the as-LOX cells at a molecular level, we investigated the response to several growth factors and the levels of their respective receptors. Among them, we found that the treatment with PDGF-BB did not produce tyrosine autophosphorylation of the PDGF-β receptor. Fig. 6*A* (*upper panel*) shows the anti-phosphotyrosine immunoblot of the immunoprecipitated PDGF-β receptor from control and as-LOX cells after 5 and 10 min of PDGF-BB treatment. It clearly appears that the tyrosine phosphorylation of the receptor occurs only in the control cells, whereas the signal is completely absent in the as-LOX cells. Surprisingly, when we challenged the same immunoprecipitate with a specific anti-PDGF-β receptor to control the efficiency of the immunoprecipitation, we could not detect a band in the as-LOX clones (Fig. 6*A*, *lower panel*). Thus, the absence of response seems to be due to a dramatic down-regulation of the receptor itself, rather than to a biochemical defect. These results prompted us to test the response of the PDGF-α receptor as well, to verify if our finding was isolated to the β receptor. Fig. 6*B* shows that also in this case there was no autophosphorylation of the receptor upon PDGF-BB triggering (*upper panel*), because of its dramatically inhibited expression (*lower panel*). The same results were obtained challenging the cells with PDGF-AA (data not shown). Moreover, we analyzed the response to another important growth factor, IGF-1, often implicated in transformation and tumorigenesis as well as in differentiation processes (28–31). Surprisingly, we detected an abnormal expression of the IGF-1β receptor, appreciably different in molecular weight and amount. Fig. 7*A* shows that

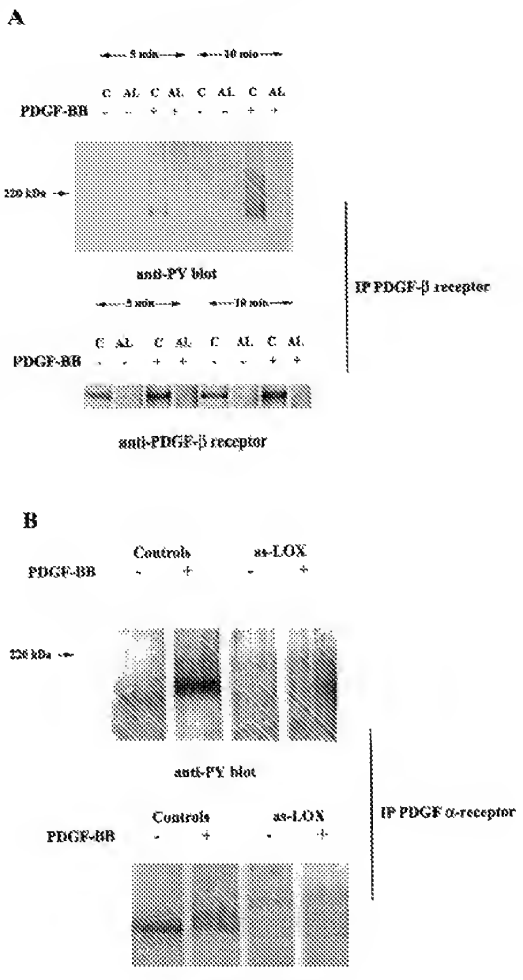

FIG. 6. **Impaired response of PDGF-β and -α receptors to PDGF-BB in as-LOX cells.** *A*, cells were starved for 48 h and then treated with 50 ng/ml of PDGF-BB for the indicated times. 500 μg of total cell lysates were immunoprecipitated with 2 μg/ml of anti-PDGF-β receptor rabbit polyclonal antibodies (Upstate Biotechnology, Lake Placid, NY). The immunoprecipitated proteins were divided into two aliquots and analyzed by Western blot on 8% SDS-PAGE with anti-phosphotyrosine monoclonal antibodies (PY-99:sc-7020, Santa Cruz Biotechnologies; *upper panel*) or anti-PDGF-β receptor rabbit polyclonal antibodies (*lower panel*). *B*, cells were starved for 48 h and then treated with 50 ng/ml of PDGF-BB for 10 min. 500 μg of total cell lysates were immunoprecipitated with anti-PDGF-α receptor rabbit polyclonal antibodies (C-20:sc-338, Santa Cruz Biotechnologies), divided into two aliquots and blotted with anti-phosphotyrosine monoclonal antibodies (PY-99; *upper panel*) or anti-PDGF-α receptor rabbit polyclonal antibodies (*lower panel*). *C*, control cells (DDM-C4); *AL*, as-LOX (DDM-AL-A4) clone. Other as-LOX clones gave the same results. The figure is representative of at least three independent experiments.

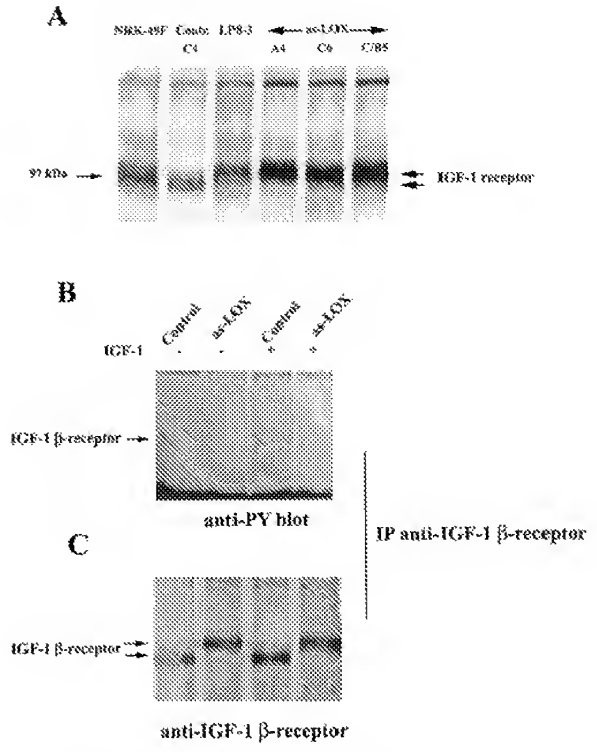

FIG. 7. **Impaired response of IGF-1β receptor to IGF-1 in as-LOX cells.** *A*, 30 μg of the indicated cell lysates were analyzed by Western blot with anti-IGF-1β receptor polyclonal antibodies (C-20:sc-713). *B*, cells were starved for 48 h and then treated with 5 ng/ml of IGF-1 for 10 min. 500 μg of total cell lysates were immunoprecipitated with anti-IGF-1β receptor polyclonal antibodies, divided into two aliquots and analyzed by Western blot with anti-phosphotyrosine monoclonal antibodies (PY-99) or *C*, anti-IGF-1β receptor polyclonal antibodies. The figure is representative of at least three independent experiments.

while NRK-49F and control-transfected cells exhibited the normal 95-kDa β receptor, the as-LOX showed a higher expression of the 105-kDa isoform. Interestingly, in the same Western blot it can be observed that the LP8–3 cell line (NIH-3T3 expressing activated Ha-*ras*) predominantly displayed the same receptor isoform. This 105-kDa variant seems to be tissue-specific and, according to some investigators, expressed during fetal development (32–35) or even in leukemic cells (36). A functional analysis of the IGF-1β receptor showed that again in as-LOX cells there was no autophosphorylation upon IGF-1 challenging (Fig. 6, *B* and *C*), although this isoform has been described as fully functional.

The down-regulation and/or absence of PDGF receptor autophosphorylation are a recurrent feature of some of the *ras*-transformed cell lines (37–39). Therefore, we analyzed the functional state of $p21^{ras}$ by using the Raf-1 Ras-Binding Domain (RBD) pull-down assay, which specifically recognizes the GTP-bound form of $p21^{ras}$. Fig. 8*A* shows that the active fraction of $p21^{ras}$ is highly increased in the two as-LOX clones that we used to induce tumors in the athymic mice. The same blot showed that the difference in the activated fraction of $p21^{ras}$ is not because of an increase in its total pool. Moreover, we studied the status of $p21^{ras}$ under different growth conditions: after 48 h of starvation, after 24 h of 10% FCS exposure following starvation, or a steady-state confluent status. Fig. 8*B* shows that the activated form of the protein is overall much higher in the as-LOX cells when compared with the control cells. Indeed, the active $p21^{ras}$ was between 25 and 11 times higher than the control cells (Fig. 8*C*), depending on their growth status. As expected, the difference was highest after 48 h of starvation, when normal cells do not show any activated $p21^{ras}$, whereas it was minimum (but still very high) after growth in 10% FCS, certainly as a result of the serum mitogenic stimulus.

Our findings prove that LOX can act as a tumor suppressor, at least in our cell model. Previous attempts to antagonize the tumorigenicity of *ras*-transformed cells by overexpressing LOX

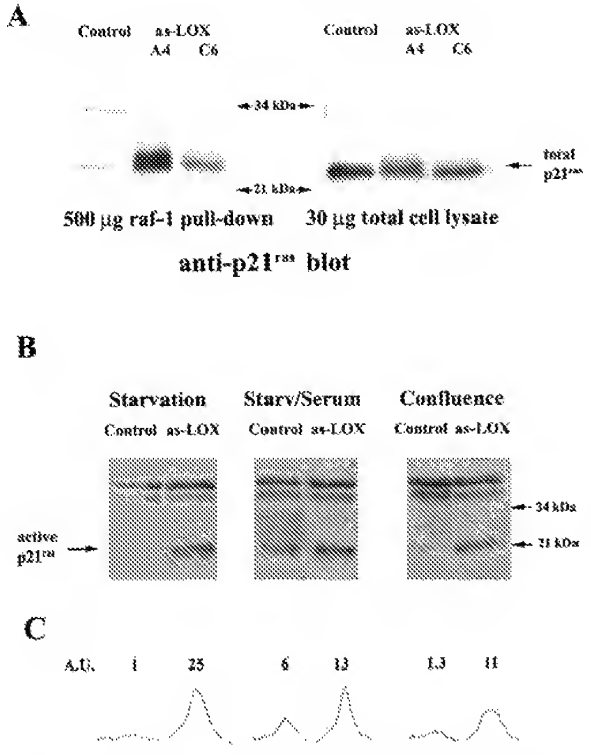

FIG. 8. **Constitutive activation of p21^{ras} in as-LOX cells.** *A, left panel*, the indicated cells were grown to subconfluence in their normal growth medium. 500 μg of total cell lysate proteins were pulled down by Raf-1 RBD-agarose resin supplied with the "Activated Ras Assay Kit", according to the manufacturer's indications (Upstate Biotechnology). The proteins separated on a 14% SDS-PAGE were then analyzed by Western blot with anti-Ras monoclonal antibodies (clone RAS10, Upstate Biotechnology, Lake Placid, NY). *Right panel*, 30 μg of the same total cell lysates were analyzed by Western blot using anti-Ras monoclonal antibodies (clone RAS10). *B*, About 8×10^6 cells/plate from each of the indicated clones were seeded in fifteen 100-mm Petri dishes and grown to subconfluence. Subsequently, they were starved for 48 h, by deprivation of FCS (*Starvation*). The cells from five Petri dishes were lysed as described in Fig. 2. The cells in the ten remnant dishes were added 10% FCS and grown for 24 h (*Starv/Serum*). The cells from five more Petri dishes were then lysed. The rest of the cells were allowed to grow to confluence and finally lysed (*Confluence*). The proteins from all the lysates were pulled down by Raf-1 RBD-agarose resin, as described above, and blotted with anti-Ras monoclonal antibodies (clone RAS10). *C*, densitometric analysis of the Western blot showed in *panel B*, using NIH Image v.1.6. The figure is representative of at least four independent experiments.

did not succeed,[2] which is in agreement with the tumor suppressor theory. The lack of tumor suppressor can trigger the transformation process, if in a permissive genetic background (*e.g.* oncogene activation), but its increase cannot overcome an ongoing tumorigenic event. Perhaps, our NRK-49F cells have a latent potential tumorigenicity, like many immortalized cell lines. In this contest, the knockdown of LOX unleashed their tumorigenic potential. Friedman and co-workers (10, 11) obtained similar results, but in a more artificial model, where the target cells, NIH-3T3, were already transformed by H-*ras* and eventually induced to revert by an interferon β/γ treatment (10). In these reverted cells, the down-regulation of LOX by antisense transfection brought the cells back to their transformed phenotype. Although very intriguing, the pre-existence of an activated p21^{ras} and the treatment with interferon-introduced variables might have influenced the results. In fact, the reversion by interferon did not affect the level of H-*ras* product, which, moreover, was under a heterologous LTR promoter. Our study confirms Friedman's findings, but in a more physiological model. Indeed, we used normal fibroblasts that did not show abnormal levels of active p21^{ras} and seemed normally regulated by the main growth factors. We also showed that as-LOX cells were unresponsive to some growth factors, the meaning of which is more difficult to explain. As mentioned above, this is not a new feature for a transformed cell line. The most obvious and rational physiological meaning of this finding might be that the pathway downstream of the growth factor receptor is already activated. Indeed, this was the case, when we look at the constitutive activation of p21^{ras} in the as-LOX cells. As elsewhere suggested, our results also reinforced the idea of a control of LOX on *ras* proto-oncogene. From our data it cannot be determined if the activation of p21^{ras} is a direct consequence of a de-regulation depending on LOX absence or is rather the indirect result of the cell transformation. Certainly, Ras activation must play an important role in the described tumorigenic process, probably enhanced by the loss of response to two important growth factors. Likely, other elements of the mitogenic pathway are activated as well, which might have triggered a negative feedback down-regulating the level of the receptors, at least for PDGF. A very recent study (40) suggests that the PDGF-β receptor can be down-regulated in an ubiquitin-dependent fashion as a consequence of loss of attachment to the substrate. We don't know yet, but a similar mechanism might be active in our as-LOX cells, because these cells display a looser attachment to the plate and, as a consequence, an elevated sensitivity to the trypsin.

Regarding the IGF-1β receptor, the meaning of the 105-kDa isoform is not clear. We believe that it is related to a sort of de-differentiation or transformation into a different cell type. Nevertheless, in this case the signal from the growth factor seems abolished. All together, our data suggest that the absence of LOX determined a sort of growth factor independence. It should be recalled that growth factors are not synonymous to mitosis, but they are rather controllers of the cell cycle, maintaining the correct equilibrium with other extracellular signals. In our case, at least two of these important controllers are lost and this, by itself, might account for the observed transformation. More studies need to be performed to analyze other elements of the signal transduction in the as-LOX cells, mainly involving the cell-substrate and cell-cell attachment, which also seem to be altered. More challenging is the goal of determining the mechanisms by which the absence of LOX induced the described alterations. New insights will certainly be revealed by the identification of putative intracellular LOX targets or substrates, one of our next goals.

[2] A. Donato, personal observations.

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